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Variation of the mitochondrial DNA and the nuclear ribosomal DNA in the striped field mouse *Apodemus agrarius* on the mainland and offshore islands of South Korea

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Abstract. Restriction fragment variations in nuclear ribosomal DNA (rDNA) spacers, and in mitochondrial DNA (mtDNA), were examined in a total of 14 individuals of the two Korean subspecies of the striped field mouse : *Apodemus agrarius coreae,* collected from the mainland and Jindo and Geoje islands, and *A. a. chejuensis* collected from Cheju Island. Analysis of heterogeneity in rDNA spacers with ten restriction enzymes, showed that the main Korean populations of *A. a. coreae* have a similar genetic background irrespective of their geographic locality. In the population from Cheju Island, however, an accumulation of a specific variation, a new SacI site within the internal spacer region of rDNA, was observed. In the contrast, analysis of heterogeneity of mtDNA with ten restriction enzymes, revealed that mtDNA haplotypes from the offshore islands were distinct from one another and distinct from those of the mainland, with up to 4% of sequence divergence, which corresponds to 1-2 million years of divergence time. It is suggested that certain geographic conditions, such as the existence of a large number of small islands, may help preserve various mtDNA haplotypes which diverged many millenial ago.

Key words. *Apodemus agrarius,* mitochondrial DNA (mtDNA), restriction fragment length polymorphism (RFLP), ribosomal DNA (rDNA), striped field mouse.

Striped field mice, *Apodemus agrarius*, are widely distributed from north-east Europe to East Asia, including the Korean Peninsula and the island of Taiwan. Two subspecies are represented in South Korea, *A. a. coreae* of the mainland

and numerous offshore islands, and the endemic *A. a. chejuensis* of Cheju Island (Cheju-do) a large island in the Korean Straits (Jones and Johnson 1965). Although genetic characterization is required to elucidate intra-specific variation, only a few reports concerning karyotypic (Tsuchiya 1984), isozymal (Tsuchiya 1984), and mitochondrial DNA (mtDNA) variation (Koh *et al.* 1993) are available at present. In the last two decades, both intra- and inter-specific genetic analysis have been performed at the DNA level, based on restriction enzyme fragment length polymorphism (RFLP), for nuclear genomic ribosomal DNA (rDNA) (Arnheim *et al.* 1980, Wilson *et al.* 1984, Hillis and Davis 1986, 1988, Suzuki *et al.* 1986, 1987, 1990, Allard and Honeycutt 1991), and for cytoplasmic mtDNA (Yonekawa *et al.* 1981, 1988, Ferris *et al.* 1983). The rRNA loci exist as a multigene family which consists of several hundred copies in the animal genome. Each repeating unit of rDNA is composed of three rRNA genes, namely those for 28S, 5.8S, and 18S RNA, which are separated from each other by spacers. The spacers are known to evolve rapidly and exhibit considerable RFLP between populations and species (Arnheim 1983). Most of the mutations, recognized by Southern blot analysis, have been fixed to yield-specific repeating unit types (repetypes) within populations or species during the course of their differentiation. Since each of the restriction sites evolves both in concert and independently (Suzuki *et al.* 1994), data for a set of variations of such sites reflects reproductive divergence of populations and such data are useful for the evaluation of genetic relationships. In contrast, variation in mtDNA occurs independently of the divergence of populations. Because of the lack of recombination between different mtDNA, and because of the lack of evidence for the existence of wandering males, in some cases a population may include considerably differentiated haplotypes, which had already diverged before the particular populations had diverged. In other cases, mtDNA may also shed light on unknown historical aspects of populations. In the case of the Japanese house mice, for example, mtDNA had an ancient haplotype prior to the invasion of the Japanese archipelago (Yonekawa *et al.* 1981, 1988), whereas in the case of house mice in Denmark, only mtDNA from other subspecies spread to the population (Ferris *et al.* 1983). In this study we compared RFLPs of both rDNA and mtDNA from several populations of two subspecies of *A. agrarius* from South Korea. From the variations in the nuclear rDNA, we concluded that although the two subspecies are clearly very closely related to one another, they are genetically different. We also discovered that there are several distinct haplotypes of mtDNA among the populations of *A. a. coreae* indicating that they have a somewhat complex evolutionary history.

Materials and Methods

1. Animals

Fourteen Korean striped field mice were collected for use in this study from eight different localities on the South Korean mainland and adjacent islands,

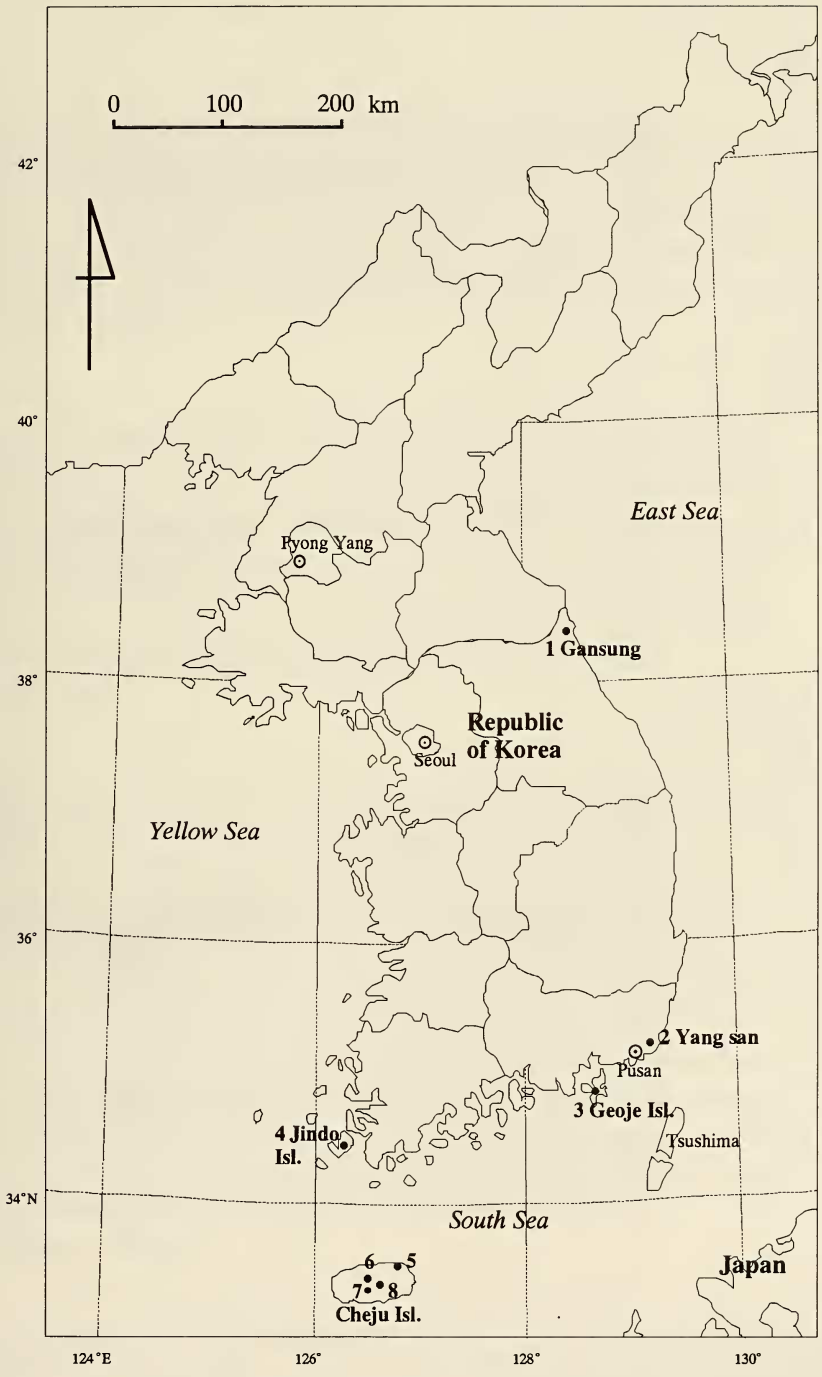

Fig. 1. Localities from which individuals *Apodemus agrarius* were collected.

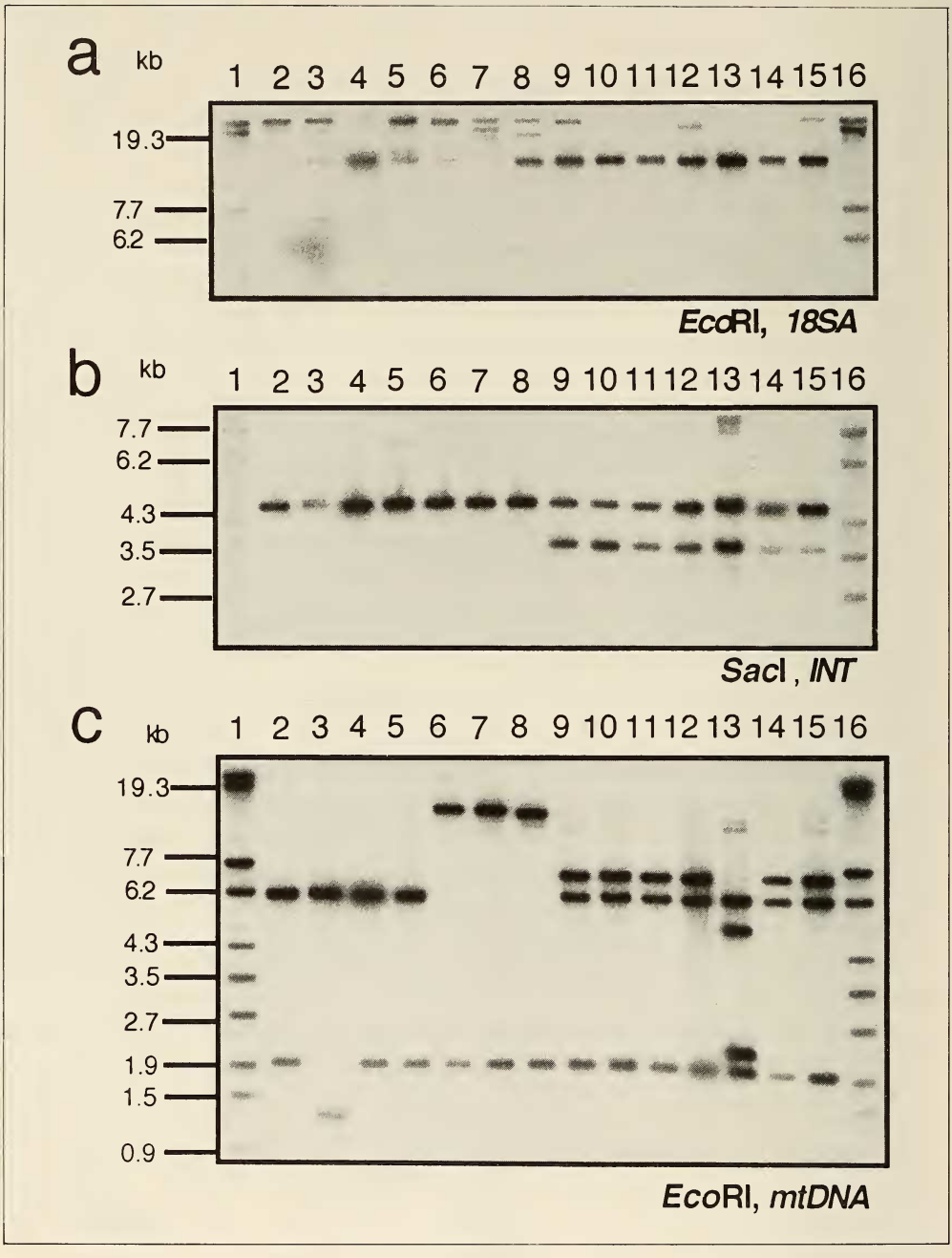

Fig. 2. Southern blot patterns of DNA cleaved with *Eco*RI (a and c), and *Sac*I (b). The probes were 0.9-kb 18SA (a and b) and whole mtDNA (c). Refer to Fig. 3 for locations of the probes of rDNA. Individuals from Gan sung (lanes 2, 3), Yang san (lanes 4, 5), Geoje (lanes 6, 7), Jindo (lanes 8), and Cheju (lanes 9-15) islands are compared. Lanes 1 and 16 show *Eco*T14I digests of λ phage DNA used as molecular markers.

the heights above sea level of these localities were 370 m, 70 m, 30 m, 30 m, 30 m, 980 m, 1280 m and 1700 m, for site numbered 1–8, respectively (see Fig. 1). On Cheju-do samples were collected from four different points.

2. Blot Analysis

Nuclear DNA was prepared from liver samples, as described by Maniatis *et al.* (1982), then southern blot analysis was carried out according to Suzuki *et al.*'s (1990) method. Genomic DNA samples were subjected to digestion with ten restriction enzymes (*Aat*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Pst*I, *Pvu*II, *Sac*I, and *Xba*I) for rDNA analysis. For mtDNA analysis, digestion was by means of *Apa*I, *Aat*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Pst*I, *Pvu*II, and *Sac*I (*Xba*I was not used). Digested DNA (on nylon filters) was hybridized sequentially with three ^{32}P-labeled rDNA probes, 18SB, 28S, and INT, and with complete mtDNA. Such sequential hybridization improves the accuracy of the measurement of fragment size, improves the confirmation of complete DNA-digestion and also minimizes laborious work as well as reducing various artificial errors. The rDNA probes (see Fig. 2) were prepared from clones of mouse rDNA, following Kominami *et al.* (1981, 1982). The mtDNA probe was prepared from rat liver, as described by Wakana *et al.* (1986).

3. Construction of Phylogenetic Trees

We began by comparing the restriction cleavage patterns between pairs of mtDNA haplotypes (Table 1) and by counting the different fragments and the fragments in common. Employing a method developed by Gotoh *et al.* (1979), in which both backward and parallel mutations are taken into account (Jukes and Cantor 1969), we were then able to produce a matrix of sequence divergence (Table 2) for all possible combinations of haplotypes (Table 1). We constructed phylogenetic trees for both the unweighted pair-group (UPGMA ; Sokal and Michener 1958) and the neighbor-joining (NJ ; Saitou and Nei 1987) methods. This was possible thanks to a computer program (NEIGHBOR in PHYLIP 3.5c) developed by Felsenstein (1993). From the information relating to the presence or absence of each restriction fragment (Table 1), we were also able to produce a phylogenetic tree for maximum parsimony. For this we used the MIX program, with a "Wagner" option, in the PHYLIP package. Confidence levels for each grouping were calculated by using a bootstrap program (SEQBOOT), with 500 replicates, in the PHYLIP package. The tree itself was produced using the CONSENCE program in the PHYLIP package.

Results

1. Heterogeneity in rDNA spacers

Examples of autoradiographic pictures of blotting with the rDNA probe 18SA can be seen in Fig. 2a and b. From the patterns of the Southern blotting, we constructed restriction maps for the coding and spacer regions of genes for rRNA (Fig. 3). These maps coincided well with the major types of rDNA

Table 1. Presence (1) or absence (0) of the 68 restriction sites of mitochondrial DNA in the ten mtDNA haplotypes in Korean striped field mice, *Apodemus agrarius*.

Haplo-type[a]	Population(s)[b] (frequency[c])	*Aat*I	*Apa*I	*Bam*HI	*Bgl*II	*Dra*I	*Eco*RI	*Hin*dIII	*Pst*I	*Pvu*II	*Sac*I
Aac1	1(1)	00110110	1100	1011	1000110	1100100011	0001100011	101010100	1001	01001	1000011
Aac2	1(1), 2(1)	10000110	1011	1011	0100111	1100100011	0001100101	101010100	1001	01001	1000011
Aac3	2(1)	10000110	1011	1011	0100111	1101000011	0001100101	101010100	1001	01001	1000011
Aac4	3(1)	00110110	1100	1100	1100100	1100100011	1000000100	100110110	1001	00111	0011111
Aac5	3(1)	01000111	1100	1100	1100100	1100100011	1000000100	100110110	1001	00111	0011111
Aac6	4(1)	10000110	1100	1100	0011011	1000001111	0100000100	100110110	0111	00111	0101011
Aah1	5(1), 7(2)	01000111	1100	1011	0010111	1100100011	0010100100	101010100	1001	10000	1000011
Aah2	6(1), 8(1)	10000110	1100	1011	0010111	1100100011	0010100100	101010100	1001	10000	1000011
Aah3	6(1)	10000110	1100	1011	0010111	1100100011	0010100100	110001001	1001	10000	1000011
Aah4	8(1)	10000110	1100	1011	0010111	0110110011	0000111100	110001001	1001	10000	1000011

[a]Aac and Aah represent haplotypes from *A. agrarius coreae* and *A. a. chejuensis*, respectively.
[b]Numbered as in Fig. 1
[c]Total number of samples observed.

Table 2. Sequence divergence among the ten mitochondrial DNA haplotypes of *Apodemus agrarius* from Korea (Upper right), on the basis of the number of common and different fragments (Lower left).

	Sequence divergence (%)[a]									
Haplotypes	Aac1	Aac2	Aac3	Aac4	Aac5	Aac6	Aah1	Aah2	Aah3	Aah4
Aac1	-	1.1	1.3	2.3	2.8	4.3	1.6	1.6	2.4	2.8
Aac2	27/11	-	0.2	2.9	2.9	3.6	1.4	1.2	1.9	2.4
Aac3	26/13	32/2	-	3.3	3.3	3.6	1.7	1.4	2.2	2.6
Aac4	22/21	20/26	19/28	-	0.4	2.4	3.1	3.1	3.8	4.4
Aac5	20/25	20/26	19/28	31/4	-	2.4	2.5	2.8	3.4	4.0
Aac6	16/33	18/30	18/30	22/22	22/22	-	3.4	3.1	3.8	4.4
Aah1	24/15	25/14	24/16	19/26	21/22	18/28	-	0.2	0.8	1.4
Aah2	24/15	26/12	25/14	19/26	20/24	19/26	30/2	-	0.6	1.2
Aah3	21/21	23/18	22/20	17/30	18/28	17/30	27/8	28/6	-	0.6
Aah4	20/25	22/22	21/24	16/34	17/32	16/34	25/14	26/12	29/6	-

[a]Sequence divergences calculated according to Gotoh *et al.* (1979).

repeating units (repetype) of *A. agrarius* previously constructed by Suzuki *et al.* (1990). Among the 26–27 restriction sites examined, these were an *Eco*RI site in the spacer upstream of the 18S rRNA gene (Fig. 2a), an *Aat*I site in the internal spacers, three were polymorphic both within and between individuals, and a *Dra*I site in the spacer downstream of the 28S rRNA gene. These kinds of polymorphism were observed in both subspecies and thus were presumed to have occured before subspecific differentiation. These polymorphic sites were likely to have been subjected to random and independent fixation processes, as observed in the natural populations of the Japanese field mouse, *A. speciosus* (Suzuki *et al.* 1994). In contrast, polymorphism in a *Sac*I site on the internal spacers was consistently and specifically observed in the genomes of individuals of *A. a. chejuensis* (Fig. 2b). Since the apparent differences between the two subspecies are confined to this variation, it may be concluded that the *A. a. coreae* and *A. a. chejuensis* have similar genomic constitutions, but have

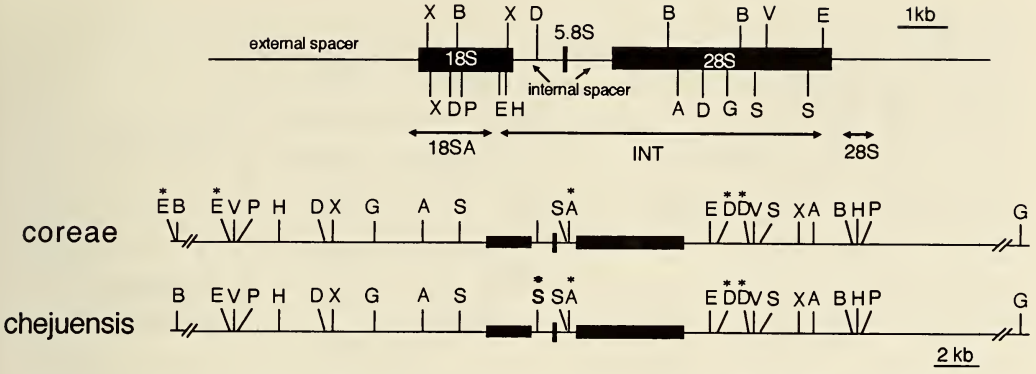

Fig. 3. Restriction maps of the major rDNA repetypes of *Apodemus agrarius coreae* and *A. a. chejuensis*. With respect to the restriction sites on the flanking spacers, only those nearest to the distal end of the genes for 18S and 28S RNA are shown. The top diagram shows the conserved restriction sites in the coding and the internal spacer regions of the gene for 18S and 28S RNA, which are not represented in the lower maps. Probe's positions are shown with arrows. Asterisks indicate polymorphic sites within and between individuals. A= *Aat*I; B=*Bam*HI; D=*Dra*I; E=*Eco*RI; G=*Bgl*II, H=*Hin*dIII; P=*Pst*I; S=*Sac*I; V= *Pvu*II; and X=*Xba*I.

differentiated substantially from each other as far as rDNA-RFLP is concerned.

2. Restriction-fragment patterns of mtDNA

Ten different haplotypes (Aac 1-6 and Aah 1-4) were found in this study (Table 1), their banding patterns, from the Southern blot analysis, with the ten restriction enzymes may be seen in Fig. 3c. There are distinct variations within this species. In particular, individuals from the two offshore islands of Jindo and Goeje, displayed different cleavage patterns from those from all other localities.

To estimate the degree of sequence divergence between haplotypes, we compared site differences between different mtDNA haplotypes. The sequence divergence among mtDNA haplotypes can be estimated from the number of common and of different restriction fragments observed (Table 2). From estimates of the amount of sequence divergence, we constructed two phylogenetic trees for mtDNA haplotypes using both the UPGMA and NJ (Fig. 4a) methods. Additionally, by considering the presence or absence of each of 68 restriction fragments (Table 1), we were also able to construct a phylogenetic tree by the maximum parsimony method (Fig. 4b). The topology of the parsimony tree was identical to that of the UPGMA tree and almost identical to that of the NJ tree. The ten haplotypes were clustered into four groups; Aac 1-3 from the Korean mainland, Aah 1-4 from Cheju-do, Aac 4 and Aac 5 from Geoje Island, and Aac 6 from Jindo Island. In contrast with the rDNA data, the mtDNA haplotypes of *A. a. coreae* were remarkably differentiated, showing the greatest sequence divergence, of 4.3%, between Aac1 and

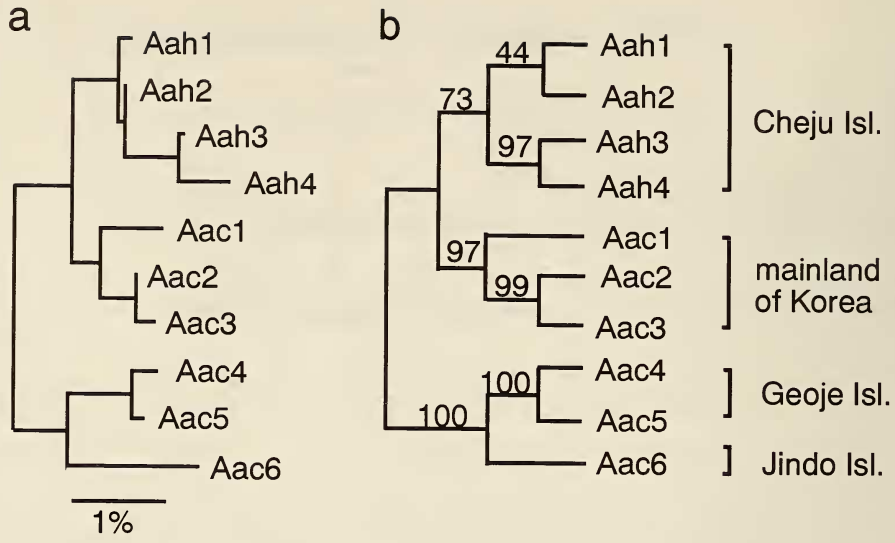

Fig. 4. NJ phylogenetic tree (a) and parsimony tree (b) for the ten haplotypes of mtDNA from *A. agrarius* collected from the Korean mainland, and from Cheju, Geoje, and Jindo islands. The bar below the NJ tree indicates 1% corrected sequence divergence. The bootstrap percentages are given for the maximum parsimony tree. Abbreviations for haplotypes are the same as in Table 1.

Aac6.

Discussion

From a molecular phylogenetic perspective, two conclusions can be drawn from our analyses of RFLP of rDNA and mtDNA. Firstly, the results of RFLP of nuclear rDNA suggest that the degree of genetic divergence within and between the two Korean subspecies of striped field mice, *A. agrarius coreae* and *A. a. chejuensis,* is low. Secondly, the results of the mtDNA RFLP revealed the presence of several distinct mtDNA haplotypes among the various populations, irrespective of their geographic distribution. These observations indicate that Korean striped field mice have similar genetic backgrounds but may have had a somewhat complex history.

From our examination of the rDNA data, we concluded that the extant Korean populations of *A. agrarius* share a similar genetic background. Two subspecies have become slightly differentiated from each other, but only one restriction site (among the 26-27 examined) was observed as a Cheju-specific variation. The new *Sac*I site was observed in approximately half the rDNA repeating units within the genomes of individuals of *A. a. chejuensis*. This level of difference is smaller than that between the two mouse subspecies, *Mus musculus domesticus* and *M. m. musculus,* in which four out of 20 sites examined have differentiated substantially (Suzuki *et al.* unpublished data). Our conclu-

sion, that the genetic backgrounds of the two Korean subspecies of *A. agrarius* are generally similar though slightly differentiated, is consistent with the conclusions of other authors. These two subspecies differ in body size (Jones and Johnson 1965) and in their electrophoretic patterns of transferrin (Tsuchiya 1984), but they are similar in karyotypes (Tsuchiya 1984). Our conclusion is also compatible with geographical evidence indicating that the final isolation of Cheju-do, from the mainland of the Korean Peninsula, occurred only 10,000–20,000 years ago (Park 1988, Ohshima 1990).

In contrast with the rDNA data, cleavage patterns of mtDNA by restriction endonuclease digestion, revealed unexpected patterns. It was found that the Korean populations of *A. agrarius* contain several distinct mtDNA haplotypes, as shown in Tables 1 and 2. Koh *et al.* (1993), working with populations from the Korean mainland, have also observed considerable differentiation in mtDNA haplotypes, ranging from 0.2% to 2.3% sequence divergence. Interestingly, our data revealed that the haplotypes of individual mice from the two offshore islands of Jindo and Geoje, were distinct from those of the mainland, even though these islands are geographically close to the mainland and thought only to have been finally isolated from the Korean Peninsula within the last 10,000 years (Park 1988). The divergence between the two different groups of mtDNA is very large, with sequence divergence of up to 4%, corresponding to divergence times of 1–2 million years, if the evolutionary rate of mtDNA is accepted to be 2–4% per million years (Wilson *et al.* 1985). It is not clear why such highly differentiated mtDNA haplotypes exist, in particular, on the offshore islands, however, there appear to be two possible explanations. Firstly, 1–2 million years ago may have already become differentiated ancestral Korean populations of *A. agrarius* and their distinctive mtDNA has merely been maintained on the offshore islands which were periodically isolated during the last ice age. During each period when the islands were connected to the Korean mainland, mtDNA haplotypes may have been mixed among individuals from the whole area of the Korean Peninsula, and then during subsequent isolation, just one mtDNA haplotype may have become fixed on each of the offshore islands. Korea has many such offshore islands and thus there are many opportunities to maintain many haplotypes of mtDNA. Secondly, it is possible that some of the distinct haplotypes may have migrated from other regions of the world. *A. agrarius* is so widely distributed that individuals from other areas may have been able to contribute to the accumulation of such extensive heterogeneity of mtDNA in Korea. Although we do not have sufficient data on mtDNA haplotypes from other pairs of the world, our preliminary investigations show, however, that these Korean haplotypes are not related to any mtDNA from individuals collected from China, Taiwan, Russia, or Germany (Suzuki *et al.* unpublished data). Thus, it seems most likely that the distinct haplotypes observed in Korea were generated there during the last ice age.

Another interesting issue is the amount of heterogeneity of mtDNA from Cheju-do. The mtDNA haplotypes from Cheju-do were related to one another,

but showed relatively high sequence divergences of up to 1.4% (Aah1 and Aah4 ; Table 2). The results indicate that mtDNA started diverging at least 0.4-0.7 million years ago. Because these forms of mtDNA are absent from the other Korean localities examined so far, it is strongly suggested that *A. agrarius* was already distributed on Cheju-do, and probably also on the Korean Peninsula, at least by the middle of the Pleistocene. It remains uncertain, however, how such divergent mtDNA haplotypes have survived on this small island of just 1819 km^2. Distinct haplotypes were even found at the same collection points, and a particular haplotype was found at several different localities. For examples, haplotype Aah 2 was collected at localty 6 (980 m above sea level) and at locality 8 (1700 m above sea level) on Mt. Halla (see Fig. 1). Thus, it may be concluded, that there are no significant biogeographic barriers on Cheju-do, and that no significant "bottle-neck event" has occurred in populations of *A. a. chejuensis* during the last half million years.

In general, mtDNA phylogeny does not always reflect the true phylogeny of either populations or species. As found in this study, mtDNA from Korean *A. agrarius* also showed such intrinsic patterns without consistency, either in the time of divergence or in geographic distribution. Our data may, however, provide some clues as to the reasons for the high degree of intra-specific mtDNA differentiation. In the case of Korean *A. agrarius,* the intrinsic geographic distribution of the mtDNA haplotypes may be due to the random dispersion of mtDNA which diverged many millenial ago, furthermore, the existence of numerous offshore islands around South Korea may have helped maintain such differentiated mtDNA. In order to clarify this issue, further examinations of samples collected from Korea, as well as samples collected from other countries are necessary.

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